The Effect of Nicotine and Cotinine on the Development of *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae)

ELISE J. HODGES, TESSA E. FUNGO, GAUTHAM, AND HANNAH D. BOWLIN

Abstract

Nicotine, readily available in electronic nicotine delivery systems, poses a lethal threat as it is easily accessible and highly toxic in its liquid form. Seventy-five percent of nicotine is metabolized into cotinine, and with the growing prevalence of nicotine-related deaths comes the increased possibility of finding nicotine or cotinine in the tissues of a corpse and thus the possibility of distorting the postmortem interval (PMI). Through entomotoxicology, the study of how drugs and toxins influence the development of insects present on a decomposing body, this experiment aimed to determine if varying levels of nicotine and cotinine affect the development of the forensically important *Cochliomyia macellaria* (Fabricius) [Diptera: Calliphoridae]. In this experiment, *C. macellaria* maggots were reared on seven varying concentrations of nicotine and cotinine and were sampled every eight hours. Length, weight and accumulated degree hours were then measured or determined. Our study found that maggots reared on lethal nicotine and lethal cotinine showed a significant decrease in length and weight as well as a delay in development time for the second instar life stage. These results suggest a relationship between high concentrations of nicotine and cotinine and the development of *C. macellaria*, which could greatly affect PMI estimations.

Introduction

By studying the rate of development and successional patterns of insects and other arthropods, forensic entomology provides scientific evidence about the arthropods in relation to a decomposing body that can be used in a court of law (Catts and Goff 1992). Forensic entomologists use this evidence to estimate how long a body has been decomposing, also known as the postmortem interval, or PMI (Catts and Goff 1992). One way to estimate PMI is to calculate Accumulated Degree Hours (ADH), which takes into account the heat units at the death scene and the time required for a specific development (Greenberg and Kunich 2002). One sub-discipline of forensic entomology is entomotoxicology, which examines the PMI-affecting relationship between drugs and toxins in a corpse as well as in insects and other arthropods present on a decomposing body (Introna et al. 2001). Some examples of drugs that have been shown to influence the development of insects on a decomposing body include drugs such as ketamine (Magni et al. 2018) and methamphetamine, which both increase the rate of development and maggot length (Magni et al. 2014, Mullany et al. 2014). However, cyclophosphamide and methotrexate (Trivia and Pinto 2018) decrease the rate of development and maggot length, which leads us to believe that nicotine could also affect the development of blowflies.

Often containing high levels of nicotine, the rise of Electronic Nicotine Delivery Systems (ENDS), has led to the fall of the cigarette business. Marketed as a transition for existing cigarette smokers, e-cigarettes and vapes are commonplace in the lives of teens and young adults due to enormously successful marketing strategies. In September 2018, the FDA released a statement saying that teen vape/e-cigarette use is at epidemic levels, with teens often obtaining vape products without ever having a history of smoking cigarettes (Commissioner 2018).

The primary delivery of nicotine in ENDS devices is e-juice (containing liquid nicotine, flavorings, glycerol, propylene glycol, and benzoic acid) heated to a vapor, which is inhaled by the user. The human body metabolizes 75% of nicotine into cotinine (Benowitz et al. 2009), which may still be present in the body a few days after stopping ingestion of nicotine (Benowitz et al. 2002); therefore, finding a body with either chemical (or both) in its system would not be uncommon. In fact, death by nicotine poisoning is on the rise due to easy access to liquid nicotine via ENDS device pods (Chatham-Stephens et al. 2014). The discrete and easily accessible nature of the refills not only leads to accidental ingestion due to mistaking it for something else, like cough syrup (Seo et al. 2016), but it also allows for purposeful self-poisoning (Bartschat et al. 2015, Solarino et al. 2010, Van et al. 2017). At the forefront of the vape frontier is the company JUUL, which occupies 75% of the e-cigarette market as of October 6th, 2018 (LaVito 2018). Its product, the JUUL, is an ENDS device with interchangeable flavored pods that have a capacity of about 200 puffs, and that comes in two concentrations of nicotine, 5% (40mg of nicotine) and 3% (23mg of nicotine) ("JUULpods and E-Liquid FAQs - JUUL Support" 2018).

Though nicotine was not shown to have an effect on the developmental time of *Calliphora vomitoria* (Linnaeus; Diptera: Calliphoridae), it has been found to shorten the length of this species of blowfly (Magni et. al 2016). However, literature searches revealed no publications examining the effects of nicotine or cotinine on the development of *Cochliomyia macellaria* (Fabricius; Diptera: Calliphoridae), also known as the secondary screwworm. The objective of this study is to examine the effects (if any) nicotine and cotinine have on the development of *C. macellaria* maggots, and the research questions are thus: 1) do nicotine and 2) cotinine have an effect on the development of *C. macellaria* maggots with regards to length, weight and developmental time? We hypothesized that both compounds would reduce length and weight while simultaneously

increasing developmental time. To test this, we reared *C. macellaria* maggots from eggs to third instar on liver homogenized with different concentrations of nicotine and cotinine matching the concentrations associated with the JUUL, along with what we determined to be the lethal doses.

Materials and Methods

Seven treatments with varying levels of nicotine and cotinine were tested. Inside each rearing container, two hundred eggs [obtained from pupae reared to oviposition from laboratory colonies maintained at Forensic Laboratory Investigative Entomological Sciences (F.L.I.E.S) Texas A&M, College Station, Texas] were placed on 100g of calf liver that was separated into two cups, each with one hundred eggs and 50g of calf liver. Using a blender (Oster Osterizer Galaxie, Sunbeam Products, Inc., Boca Raton, Florida), calf liver was homogenized with the respective concentration of nicotine or cotinine for each treatment using a stock solution (Appendix); the control liver was blended to mimic treated livers. In separate rearing chambers (Figure 3), the treated liver (in a plastic cup) was placed on a 2.5cm-deep layer of vermiculite in a 35.6x20.3x12.4cm (5.7L) shoe box container (Sterilite Corporation, Townsend, Massachusetts). Cheesecloth was placed over the bin and secured with an elastic band. All treatments were placed in a Percival I29VLXC8 Incubator with a 14:10 photoperiod. The temperature (27°C) and relative humidity (70%) were kept constant throughout the experiment, with Thermochron iButtons (Embedded Data Systems, Lawrenceburg, Kentucky) inside to detect temperature and relative humidity (RH).

Treatments. Seven different treatments were tested: control (CT), lethal nicotine (LN), lethal cotinine (LC), 5% nicotine (5N), 5% cotinine (5C), 3% nicotine (3N), and 3% cotinine (3C) (see appendix for concentrations). The lethal dose of nicotine, 6.5mg/kg, is based on the range

(6.5-13mg/kg) determined from the lethal dose for nicotine in dogs, who exhibit a similar reaction to nicotine to that of humans (Mayer 2014).

Destructive Sampling. Once the eggs were placed on the liver, sampling started approximately 8-10 hours later starting at 0700, then again at 1500 and 2300. While sampling, the three largest, most visible maggots from each treatment were collected, boiled and preserved in 70% ethanol. Using a scale (Fisher-Scientific A-160, Fisher Scientific International, Inc., Hampton, New Hampshire) individual weights of maggots were determined. Each maggot's length was measured using digital measuring software [Olympus cellSens software (Olympus Corporation, Shinjuku, Tokyo, Japan)] and an Olympus SZ61 microscope with an Olympus SC30 camera (Olympus Corporation). Using a Leica S7E microscope (Leica Microsystems GmbH, Wetzlar, Germany), the maggots' instar was determined by observing the number of slits per spiracle. Destructive sampling ceased once maggots abandoned the liver source. Observations on pupation and emergence on adult flies were recorded, however.

Statistical Analysis. The developmental effects of nicotine and cotinine in *C. macellaria* larvae in terms of length and weight, with respect to different treatments, were analyzed using one-way, repeated measures analysis of variance (ANOVA). To analyze the differences and variations in development time for the nicotine and cotinine treatments, a Chi-square test of independence was used ($P < 0.05$).

Results

Cochliomyia macellaria maggots were reared on calf liver treated with varying concentrations of nicotine and cotinine. Length, weight and accumulated degree hours (ADH) were used to determine the rate of development of these maggots. Maggots that ingested liver from the lethal nicotine (LN) and lethal cotinine (LC) treatments demonstrated decreased length and

weight, along with delayed development time between instars, when compared to the control (CT). Predominantly, maggots from the 5% nicotine (5N), 5% cotinine (5C), 3% nicotine (3N), and 3% cotinine (3C) treatments had lengths and weights that were similar to the CT (Figs. 1, 2, and Table 1).

There is a concentration effect of nicotine on maggot development. In Fig. 1a, maggots from LN were 1.37mm shorter than those from the CT in the span of 184 hours (5.15mm vs. 6.52mm); while maggot lengths from treatments 5N and 3N did not differ from those in the CT, respectively. The same trend is seen in Fig. 2a, where maggots that ingested LN demonstrated decreased weights in comparison to the maggots that ingested CT liver (0.0124g vs. 0.032g), while maggots from treatments 5N and 3N still did not differ greatly from the maggots in the CT, respectively. In Table 1, the development time for the 1st instar life stage for the maggots reared on LN was delayed by eight hours, while the maggots reared on 5N showed a development time that was sped up by eight hours, and the maggots from the 3N treatment did not differ from the control. For the 2nd instar life stage, the development time for maggots from the LN treatment was greatly delayed in comparison to the maggots from the CT (64 hours vs. 16 hours), respectively. The development time of the maggots from treatment 5N was also not as delayed as those in LN when compared to the maggots from CT (48 hours vs. 16 hours), and the same trend is seen with the maggots from 3N and the CT (40 hours vs. 16 hours), respectively.

There also is a concentration effect for cotinine, which is similar to the findings for the nicotine treatments. In Figure 1b, maggots that ingested LC tended to have decreased lengths in comparison to the maggots from the CT in the span of 184 hours (5.21mm vs. 6.52mm), while the maggots from treatments 5C and 3C followed the same general trend as the maggots from the CT, respectively. When comparing the weights in Figure 2b, maggots from LC demonstrated a 0.0174g

decrease in weight in the span of 184 hours (0.0146g vs. 0.032g), respectively. Maggots from treatments 5C and 3C did not differ from the maggots in the CT, although the 3C maggots tended to have decreased weights in comparison to the CT (0.0194g vs. 0.032g) in the span of 184 hours, respectively. In Table 1 for the 1st instar life stage, maggots reared on LC and 3C development times were delayed to 32 hours in comparison to the CT (24 hours), while maggots reared on 5C was shortened to 16 hours, respectively. Table 1 also shows that for the 2nd instar life stage, maggots from the LC treatment's development time was greatly delayed (64 hours vs. CT 16 hours), while maggots from 5C also demonstrated a delayed development time (48 hours vs. 16 hours, respectively), and maggots from 3C, too, demonstrated a delay in developmental time (56 hours vs. 16 hours, respectively).

There were two statistical tests utilized to analyze the length, weight, and ADH data. For the statistical analyses for length and weight (Figs. 1 and 2), a repeated-measure one-way ANOVA was used to compare the means. For length (Fig. 1a and b), the ANOVA showed significant differences between the CT, LN and LC (F=18.851, df=6, P<0.05). No significant difference was found between the CT and 5N, 3N, 5C, and 3C (F=18.851, df=6, P>0.05). For weight (Fig. 2a and b), the ANOVA also showed significant differences between CT, LN and LC (F=6.297, df=6, P<0.05). Still, no significant difference was found between the CT and 5N, 3N, 5C, and 3C (F=6.297, df=6, P>0.05). For the statistical analyses for ADH (Table 1), a Chi-square test of independence was used to compare the nicotine and cotinine treatments to the CT. Four total Chi-square values were calculated, two for the 1st instar life stage for nicotine and cotinine treatments, and two for the 2nd instar life stage for nicotine and cotinine treatments. When comparing the nicotine treatments (LN, 5N, and 3N) to the CT during the 1st instar life stage, no significant results were found ($x^2_{(3)}$=5.33, P>0.05). However, when comparing the cotinine treatments (LC, 5C, 3C)

to the CT during the 1st instar life stage, these results were significant ($x^2_{(3)}$=8, P<0.05). For the 2nd instar life stages for the nicotine treatments, significant results were found ($x^2_{(3)}$=244, P<0.05). The results for the cotinine treatments during the 2nd instar life stage were also significant ($x^2_{(3)}$=308, P<0.05).

Discussion

The toxicological effects on *Cochliomyia macellaria* maggots reared on calf liver contaminated with different concentrations of nicotine and cotinine showed that 1) maggots grown on higher concentrations of nicotine and cotinine (LN and LC) were observed to be significantly shorter in length and weighed less when compared to the control (CT), 2) no significant differences were observed between first to second instar accumulated degree hours (ADH) for nicotine, yet all cotinine treatments were found to be significant when compared to the control (CT), 3) all treatments (LN, LC, 5N, 5C, 3N, and 3C) were observed to be significant (P <0.05) in development time between second and third instar. Therefore, the results suggest a relationship between high levels of nicotine and cotinine and the overall development of *C. macellaria* maggots, which is vital because both length and ADH are accepted ways to calculate PMI.

Maggot development affected by specific concentrations of drugs has been previously tested in other studies, including the amount of drugs present in the maggots. For instance, Campobasso et al. (2004) found that drugs such as cocaine, barbiturates, opiates, and antidepressants were present in samples of human liver, yet when testing maggots collected from those samples, the drugs were found in lower concentrations. This could suggest that the maggots are ingesting the drugs they have been reared on. Magni et al. (2016) also found that higher concentrations of nicotine in the liver resulted in a higher rate of detection of nicotine compounds when gas chromatography-mass spectrometry (GC-MS) was performed. Although more nicotine

compounds were detected in GC-MS as the concentration of the drug increased, it did not necessarily correlate with the concentration used to treat the liver. When testing codeine accumulation in *Lucilia sericata,* Kharbouche et al. (2008) found only the flies reared on the highest concentrations of morphine (2.0 mg/kg and 30 mg/kg) showed morphine at all stages of development when tested using GC-MS. Furthermore, no morphine was detected in pupal cases (Kharbouche et al. 2008). In more cases, drugs identified through GC-MS only showed either the absence or presence of the drug in question, but was unable to quantify the drug's concentration (Notle, Pinder and Lord 1992, Definis-Gojanović 2007).

When maggots feed on a contaminated liver or a decomposing body, maggots will either bioaccumulate or excrete the ingested drugs, possibly affecting their length, weight, and development time (Carvalho et al. 2001). Carvalho et al.'s (2001) study on diazepam on *Chrysomya albiceps* (Wiedemann) and *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae) show an affinity for bioaccumulation as there was a significant influence of diazepam on all stages of maggot development, specifically an increased time for pupariation and fly emergence, and diazepam was detected in pupae and adult flies. Our results indicate that the development time of the maggots from the second to third instar for both nicotine and cotinine treatments show significant differences between the control, along with the length and weight for the lethal doses. Because of this, we are led to believe that nicotine and cotinine bioaccumulate in *C. macellaria*. In addition to bioaccumulation, this could partially be due to the size principle, where a maggot can only eat so much during each instar before its cuticle must be shed, and since more drugs are consumed between second and third instar, it has a greater effect on their development during that period (Chapman 1971). GC-MS would further determine whether nicotine and cotinine are still present through the entire lifespan of *C. macellaria* flies from their larval state through adulthood

to the next generation, which could be used to determine if they bioaccumulate or excrete those drugs.

Multiple limitations became obvious over the course of this experiment, some of which can be adjusted for future experimentation. In the future, better sampling methods are needed to collect maggot samples while disturbing them as little as possible. For example, third instar maggots were collected from treatment one at 48 hours, yet the next time a third instar was collected was at 64 hours. Maggots had to be collected from the top and only exposed by a soft squeezing of the sides of the cup, so our sampling was not representative of what was truly in each liver cup. Also, a large surface area of the liver, such as multiple liver cups in the containers or flatter liver cups, may help with consistent sampling. As for the liver, when homogenized it activates the enzyme Cytochrome P450 2A6 (CYP2A6), which breaks down nicotine into cotinine and possibly has a post-mortem effect (Magni et al. 2016). This could break down the nicotine before the maggots finish eating, thus causing the maggots to ingest cotinine. Performing GC-MS would help determine whether CYP2A6 has an effect on nicotine in the liver and whether or not the maggots are in fact eating liver treated with nicotine. Beyond this, GC-MS would help answer the question of bioaccumulation or excretion.

In conclusion, higher concentrations of nicotine and cotinine had a significant impact on length, weight, and ADH when lethal doses of nicotine (LN) and cotinine (LC) were compared to the control (CT). Consequently, these findings could potentially distort the PMI when not taken into account, which could potentially lead to the incrimination of the wrong person. Furthermore, our results emphasize a need for future experiments and improvements to the existing methods on how to factor in the effects of drugs while calculating PMI. Moreover, the addition of the GC-MS

component to this study would further enhance and strengthen our results by quantifying the amount of nicotine and cotinine present in the maggots.

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References Cited

Bartschat, S., K. Mercer-Chalmers-Bender, J. Beike, M. A. Rothschild, and M. Jübner. **2015**. Not only smoking is deadly: fatal ingestion of e-juice—a case report. Int. J. Legal Med. 129: 481–486.

Benowitz, N. L., P. Jacob III, K. Ahijevych, M. J. Jarvis, S. Hall, J. LeHouezec, A. Hansson, E. Lichtenstein, J. Henningfield, J. Tsoh, R. D. Hurt, and W. Velicer. **2002**. Biochemical verification of tobacco use and cessation. Nicotine Tob. Res. 4: 149–159.

Benowitz, N. L., J. Hukkanen, and P. Jacob. **2009**. Nicotine Chemistry, Metabolism, Kinetics and Biomarkers. Handb. Exp. Pharmacol. 29–60.

Campobasso, C., M. Gherardi, M. Caligara, L. Sironi, and F. Introna. **2004**. Drug analysis in blowfly larvae and in human tissues: a comparative study. International Journal of Legal Medicine. 118.

Carvalho, L. M. L., A. X. Linhares, and J. R. Trigo. **2001**. Determination of drug levels and the effect of diazepam on the growth of necrophagous species of forensic importance in southeastern Brazil. Forensic Science International. 5.

Catts, E. P., and M. L. Goff. **1992**. Forensic Entomology in Criminal Investigations. Annu. Rev. Entomol. 37: 253–272.

Chapman, R. F. 1971. The insects: structure and function, 2nd ed. pp. 436. Elsevier, New York, NY.

Chatham-Stephens, K., R. Law, E. Taylor, P. Melstrom, R. Bunnell, B. Wang, B. Apelberg, and J. Schier. **2014**. Notes from the Field: Calls to Poison Centers for Exposures to

Electronic Cigarettes (Weekly Report). Morbidity and Mortality Weekly Report. 63(13): 292-293.

Commissioner, O. of the. **2018**. Press Announcements - Statement from FDA Commissioner Scott Gottlieb, M.D., on new steps to address epidemic of youth e-cigarette use. (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm620185.htm).

Greenberg, B., and J. C. Kunich. 2002. Entomology and the Law: Flies as Forensic Indicators. Cambridge University Press, 2nd edition.

Introna, F., C. Campobasso, and M. Goff. **2001**. Entomotoxicology.

JUULpods and E-Liquid FAQs - JUUL Support. **2018**. JUULpods and E-Liquid FAQs - JUUL Support. (https://support.juul.com/home/learn/faqs/juulpod-basics).

Kharbouche, H., M. Augsburger, D. Cherix, F. Sporkert, C. Giroud, C. Wyss, C. Champod, and P. Mangin. **2008**. Codeine accumulation and elimination in larvae, pupae, and image of the blowfly *Lucilia sericata* and effects on its development. International Journal of Legal Medicine. 122: 205–211.

LaVito, A. 2018. Juul e-cigarette sales have surged over the past year. (https://www.cnbc.com/2018/07/02/juul-e-cigarette-sales-have-surged-over-the-past-year.html).

Magni, P. A., T. Pacini, M. Pazzi, M. Vincenti, and I. R. Dadour. **2014**. Development of a GC–MS method for methamphetamine detection in *Calliphora vomitoria* L. (Diptera: Calliphoridae). Forensic Sci. Int. 241: 96–101.

Magni, P. A., M. Pazzi, M. Vincenti, E. Alladio, M. Brandimarte, and I. R. Dadour. **2016**. Development and validation of a GC–MS method for nicotine detection in *Calliphora vomitoria* (L.) (Diptera: Calliphoridae). Forensic Sci. Int. 261: 53–60.

Magni, P. A., M. Pazzi, J. Droghi, M. Vincenti, and I. R. Dadour. 2018. Development and validation of an HPLC-MS/MS method for the detection of ketamine in *Calliphora vomitoria* (L.) (Diptera: Calliphoridae). J. Forensic Leg. Med. 58: 64–71.

Mayer, B. 2014. How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century. Arch. Toxicol. 88: 5–7.

Mullany, C., P. A. Keller, A. S. Nugraha, and J. F. Wallman. **2014**. Effects of methamphetamine and its primary human metabolite, p-hydroxymethamphetamine, on the development of the Australian blowfly *Calliphora stygia*. Forensic Sci. Int. 241: 102–111.

Nolte, K. B., R. D. Pinder, and W. D. Lord. **1992**. Insect Larvae Used to Detect Cocaine Poisoning in a Decomposed Body. Journal of Forensic Sciences. 37: 13304J.

Seo, A. D., D. C. Kim, H. J. Yu, and M. J. Kang. **2016**. Accidental ingestion of E-cigarette liquid nicotine in a 15-month-old child: an infant mortality case of nicotine intoxication. Korean J. Pediatr. 59: 490–493.

Solarino, B., F. Rosenbaum, B. Rießelmann, C. T. Buschmann, and M. Tsokos. **2010**. Death due to ingestion of nicotine-containing solution: Case report and review of the literature. Forensic Sci. Int. 195: e19–e22.

Sutlović, D., and M. Definis-Gojanović. **2007**. Fatal Poisoning By Alcohol and Heroin. Archives of Industrial Hygiene and Toxicology. 58.

Trivia, A. L., and C. J. de C. Pinto. **2018**. Analysis of the Effect of Cyclophosphamide and Methotrexate on *Chrysomya megacephala* (Diptera: Calliphoridae),. J. Forensic Sci. 63: 1413–1418.

Van, D. der M., A. D. Pranger, I. Jansen, E. B. Wilms, H. Kieft, and J. G. Maring. **2017**.

[Fatal intoxication with nicotine for e-cigarette]. Ned. Tijdschr. Geneeskd. 161: D1591–D1591.

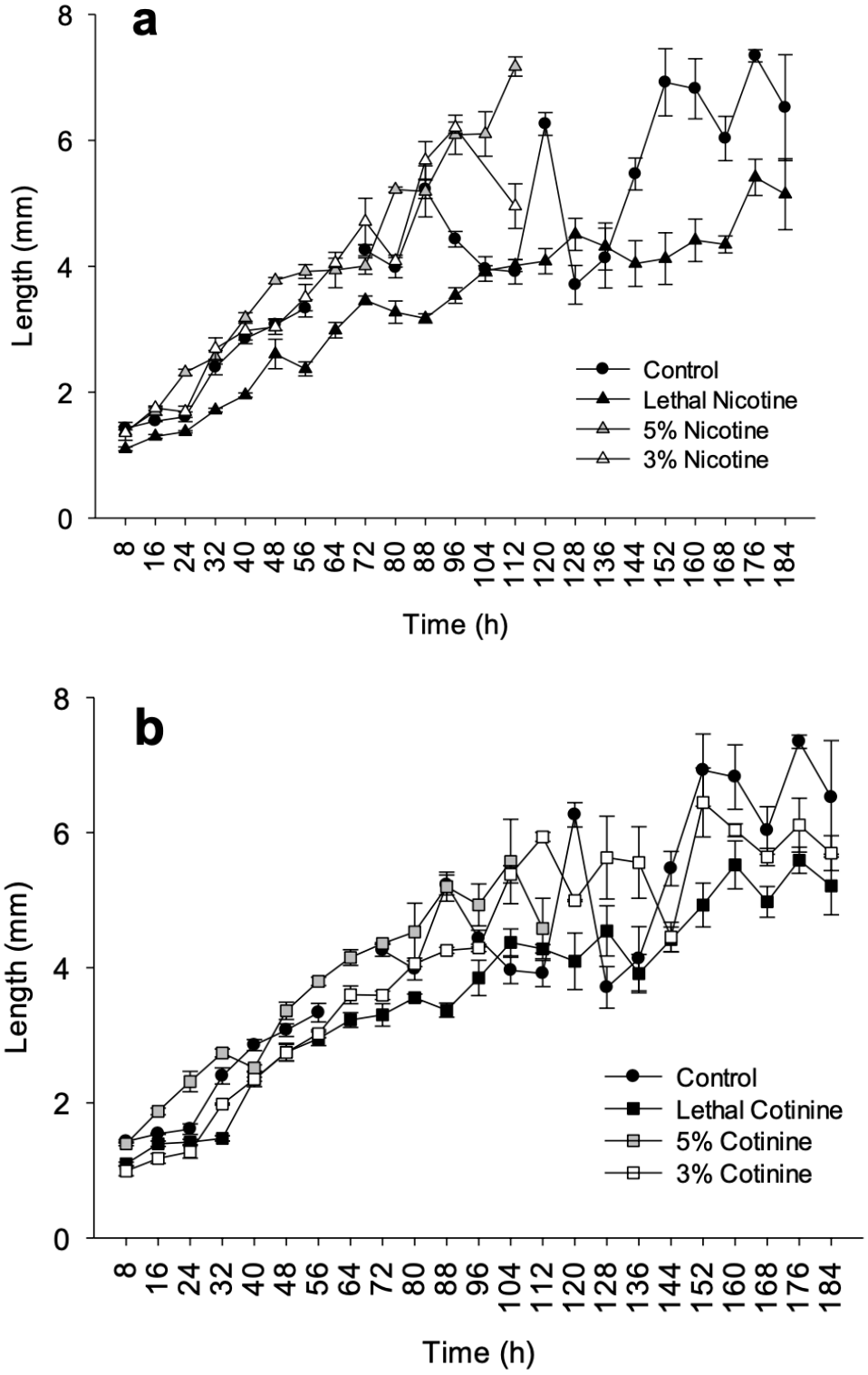

Fig. 1. Mean length (*n*=4) ± SE of *Cochliomyia macellaria* maggots reared at 27°C on liver treated with varying concentrations nicotine (a) and cotinine (b) over time. In graph (a), the data collection for 5N and 3N ceased at 112 hours. In graph (b), data collection for 5C ceased at 112 hours.

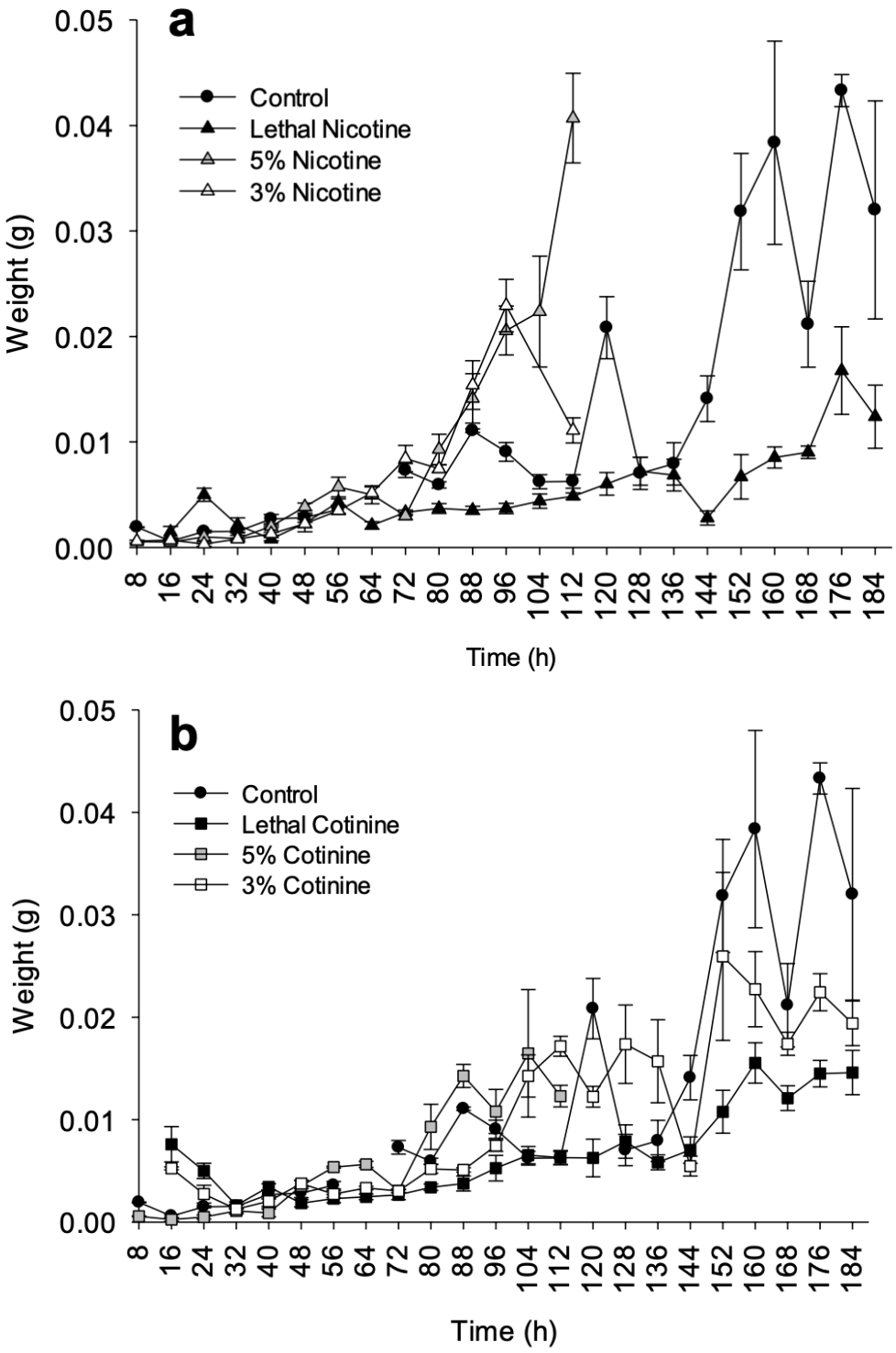

Fig. 2. Mean weight (*n*=4) ± SE of *Cochliomyia macellaria* maggots reared at 27°C on liver treated with varying concentrations nicotine (a) and cotinine (b) over time. In graph (a), the data collection for 5N and 3N ceased at 112 hours. In graph (b), the data collection for 5C ceased at 112 hours.

Table 1. Accumulated Degree Hours (ADH) until the first collection of second and third instars of *Cochliomyia macellaria* maggots, counted from first collection (Hour 0) from all treatments. Maggots were reared at a temperature of 27°C and a relative humidity of 70.8%.

Duration (hours)							
Life Stage	**Control**	**Lethal Nicotine**	**Lethal Cotinine**	**5% Nicotine**	**5% Cotinine**	**3% Nicotine**	**3% Cotinine**
1st Instar	24	32	32*	16	16*	24	32*
2nd Instar	16	64*	64*	48*	48*	40*	56*

***** = this value is significant via a Chi-square test of independence.

APPENDIX

Treatment	µL of solution
C	0
LN	32.19
LC	73.12
5N	2.90
5C	6.59
3N	1.67
3C	3.795

For this experiment, two rounds of calculations were made. The first round was to determine the lethal dose for nicotine for a 68kg person, a .05kg liver, the percentage of the lethal dose for the 5 and 3% JUULs, and the milligram (mg) values for nicotine and cotinine on a 50g liver. The second round of calculations, treatment microliter (µL) calculations, used the treatment mg calculations to determine a specific volume of nicotine and cotinine stock solution to treat the 50g of calf liver with.

Treatment Milligram Calculations

The lethal dose of nicotine is based on the weight of the person ingesting the chemical, so these calculations are based on the assumption of a 68.18kg person consuming one JUUL pod (5% and 3%) in one day.

5% JUUL: Contains 40mg of nicotine

3% JUUL: Contains 23mg of nicotine

Lethal dose for 68.18kg person (Mayer 2014):

6.5mg/kg x 68.18kg = 443.18mg of nicotine to kill a 150lb person.

Finding the percentage of the lethal dose for the 40mg JUUL (5%):

40mg / 443.18mg = .09025 x 100 = 9.025% A 40mg JUUL is ~9% of the lethal dose for this person.

The same thing is done for the 23mg JUUL (3%).

23mg / 443.18mg = .0519 x 100 = 5.1897% A 23mg JUUL is ~5% of the lethal dose for this person.

Determining the lethal dose for the quantity of liver used in this experiment, 50g:

6.5mg/kg x .05kg = .325mg of nicotine is the theoretical lethal dose for a .05kg liver, and is the nicotine concentration for LN.

.09025 x .325mg = .02933mg. This would be the equivalent of the liver using the 40mg (5%) JUUL, and is the nicotine concentration for treatment 5N.

.05187 x .325mg = .016867mg. This would be the equivalent of the liver using the 23mg (3%) JUUL and is the nicotine concentration for treatment 3N.

For the cotinine concentrations, these values were multiplied by .75 (75%) because 75% of nicotine is metabolized into cotinine (Benowitz et al. 2009):

LC; lethal cotinine: .325mg x .75 = .24375mg of cotinine

5C; 5% cotinine: .02933mg x .75 = .022mg of cotinine

3C; 3% cotinine: .01687mg x .75 = .01265mg of cotinine.

Stock Solutions and Treatment µL Calculations

For nicotine, 5mL of 99+% L-Nicotine solution was used to make a stock solution because the µL amounts for each treatment were too small to be used with a micropipette. In order to combat this, a 1:100 stock solution was made that had 1mL of the 99+% L-Nicotine and 99mL of deionized water. This is the stock solution that was used to treat the liver for treatments LN, 5N, and 3N. The formula, volume = mass/density was used to determine the volumes (µL). Density of nicotine: 1009.7mg/mL

Nicotine Treatments:

LN; lethal nicotine: volume = .325mg / 1009.7mg/mL = .0003218mL = .32187µL

5N; 5% nicotine: volume = .02933mg / 1009.7mg/mL = .000029 mL = .029µL

3N; 3% nicotine: volume = .01687mg / 1009.7mg/mL = .000016708mL = .01671µL

Note: Because a 1:100 stock solution was made, 32.19µL, 2.90µL, and 1.67µL were the volumes that were used to treat the liver for each nicotine treatment.

For cotinine, 50mg of solid (-)-Cotinine was ground up with a pestle in a bowl. Fifteen mL of deionized water was added and the cotinine was incorporated, to make a 0.0189M solution. In order to calculate the µL volumes to extract from this stock solution, the treatment milligram calculations for cotinine were converted into moles. The formula, Molarity = moles / Liter was used to find the µL volumes.

Cotinine Treatments:

LC; lethal cotinine: L = 1.383x10^-6 mol / .0189M = 7.312x10^-5 L = 73.12µL

5C; 5% cotinine: L = 1.25x10^-7 mol / .0189M = 6.59x10^-6 L = 6.59µL

3C; 3% cotinine: L = 3.795x10^-8mol / .0189M = 3.795x10^-6 L = 3.795µL

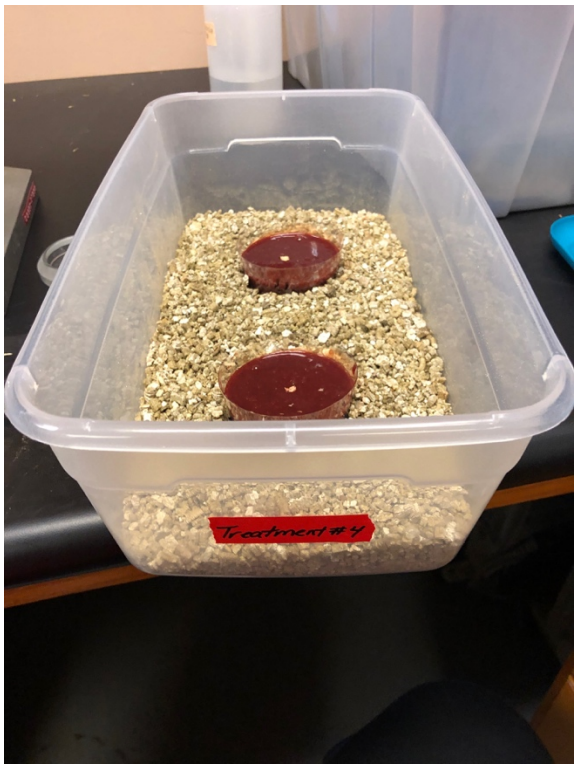

Fig. 3. *A representative example of the treatment chamber containing two cups of 50g of treated liver for LN with ~100 eggs on top of the liver. Two cups of liver placed on 2.5cm of vermiculite in a plastic shoebox. For storage, cheesecloth was placed over the box and secured with an elastic band. A shoebox lid was then placed on top of this with holes to allow for air flow.*